#12 Declaration 7/24/01

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of: Kozikowski *et al.*

Serial No.: 09/246,307

Group Art Unit: 1654

Filed: February 8, 1999

Examiner: Anish Gupta

For: CYCLIC DIPEPTIDES AND AZETIDINONE COMPOUNDS AND THEIR USE IN TREATING CNS INJURY AND NEURODEGENERATIVE DISORDERS

Attorney Docket No.: 9328-009-999

DECLARATION OF ALAN I. FADEN
UNDER 37 C.F.R. § 1.132

Assistant Commissioner for Patents
Washington, D.C. 20231

Sir:

I, **ALAN I. FADEN**, declare that:

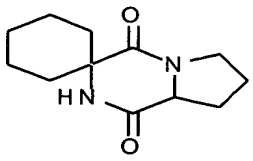
1. I am a co-inventor of the subject matter disclosed and claimed in the above-identified patent application.

2. I currently hold the position of Professor of Neuroscience, Neurology and Pharmacology at Georgetown University Medical Center, Georgetown University, Washington D.C., the assignee of the above-identified application, where I have been employed since 1991. I received an M.D. from the University of Chicago in 1971, and I have been working in the field of neurology since 1972. To date, I have published my research in

more than 300 papers and chapters. My technical experience and selected publications are summarized in my *Curriculum Vitae*, which is appended hereto as Exhibit B.

3. I have reviewed the above-identified patent application and the outstanding Office Action mailed January, 17, 2001. I understand that the Patent Office has rejected Claims 12-17, 21-28, 31, 32 and 73-81 under 35 U.S.C. §112, first paragraph for lack of enablement.

4. In addition to the experiments described in the Specification and my Declaration under 37 C.F.R. § 1.132 of November 2, 1999, I have supervised an additional experiment using a compound disclosed in the instant Application. This additional experiment further establishes that the inventions recited in pending Claims 12-17, 21-28, 31, 32 and 73-81 are fully enabled. The compound used in the assay described in this affidavit has the same designation that was provided in the specification. Namely,



2(a)

5. I supervised an experiment to test the ability of compound 2(a) to protect neuronal cultures incubated with beta amyloid from apoptotic cell death. The experiment described in this affidavit was conducted using neurons prepared as described below. Assessment of neuronal cell death was assayed by measuring lactase dehydrogenase (LDH), as described below.

Preparation of Neurons and Addition of Beta-amyloid in Presence or Absence of 2(a)

Neurons were prepared from 18 d Sprague-Dawley rat embryonic cortices *via* trituration of dissociated cortices in Hank's salts without calcium of magnesium. Isolated cells were suspended in neurobasal medium (Life Technologies, Grand Island, NY) supplemented with 25 μM glutamate (Sigma, St. Louis, MO), 0.5 mM glutamine (Sigma, St. Louis, MO), 1% antibiotic anitmycotic solution and 2% B27 supplement (Life Technologies, Grand Island, NY). Cells were seeded at 1 x 10^6 cells/ml onto microplates precoated with 10 μg/ml poly-D-lysine (Sigma, St. Louis, MO). Cultures were incubated at 37 °C in a humid atmosphere with 4% CO_2 and were fed after four days *in vitro* (DIV) with neurobasal medium supplemented with 0.5 mM glutamine, 1% antibiotic-anitmycotic solution and 2% B27 supplement. Experiments were performed at 11-14 DIV. 50 μM of 25-35 beta-amyloid was added to culture media in the presence or absence of 2(a) (0.1 μM concentration). Additional 2(a) was added at 12 hour intervals until 48 hours after the addition of beta-amyloid. At this point the cells were inspected visually and assayed for lactate dehydrogenase activity.

Assessment of Effect of 2(a) on Cell Death in the Presence of Beta-Amyloid.

Cell death was assessed 48 hours after introduction of 50 μM 25-35 beta-amyloid *via* lactate dehydrogenase (LDH) assay. In the assay described in this affidavit, fifty percent of the media was removed and diluted 1:3 with LDH assay reagent (5 mM β-NAD, 25 mM lactic acid, 0.3% bovine serum albumin, 100 mM TRIS, 0.9% NaCl, pH 8.45). Optical density at 340 nM was measured over 250 seconds at five second intervals for a total of fifty readings. The slope of the absorbance curves generated over 250 seconds represented LDH activity. Results reflect the difference between LDH release induced by the presence of beta-

amyloid and LDH released in the absence of beta-amyloid. LDH data is expressed as percentage of LDH released from injured controls (means ± SEM). LDH values from non-injured controls were averaged and subtracted from all dat points. Bars represent the means ± standard error measurements (SEM) for *n*-10-12 wells per condition. Results, as shown in Exhibit C, indicate that compound 2(a) at the relatively low concentration of 0.1 μM significantly attenuates apoptotic cell death (as reflected by LDH release) caused by beta-amyloid in neuronal cultures. Accordingly, compound 2(a) provides significant neuroprotection to neurons incubated in the presence of beta-amyloid, which is well-known in the art to cause apoptotic cell death in neurons and has been strongly implicated in the art as a causative factor in Alzheimer's disease.

6. I believe this experiment along with the experiments described in my Declaration under 37 C.F.R. § 1.132 of November 2, 1999 demonstrate that the compounds of the invention provide significant neuroprotection from excitotoxic injury, ischemic injury, traumatic injury, necrotic injury, apoptotic cell death caused by staurosporine and apoptotic cell death caused by beta-amyloid. Accordingly, I believe that the Specification of the instant Application fully enables the method of neuroprotection and the method of cognition enhancement recited in presently pending Claims 12-17, 21-28, 31, 32 and 73-81.

7. I affirm that all statements made in this Declaration are of my own knowledge and are true and that all statements made on information and belief are believed to be true and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title

18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issuing thereon.

Dated: 7/9/01

ALAN I. FADEN, M.D.